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ISOLATION AND SEQUENCE ANALYSIS OF A PIGMENT-DISPERSING HORMONE FROM EYESTALKS OF THE CRAB, *CANCER MAGISTER*

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ABSTRACT

The application of separation procedures, including liquid partition, gel filtration, ion-exchange chromatography, partition chromatography, and reversed-phase high-performance liquid chromatography, enabled the purification of an octadecapeptide pigment-dispersing hormone (PDH) from powdered lyophilized eyestalks of the crab, *Cancer magister.* Automated Edman degradation, followed by the identification of carboxyl-terminal amide, established the sequence of the peptide as: Asn-Ser-Glu-Leu-Ile-Asn-Ser-Ile-Leu-Gly-Leu-Pro-Lys-Val-Met-Asn-Asp-Ala-NH_2. The characterized *Cancer* PDH is structurally identical to an octadecapeptide PDH of *Uca pugilator,* but differs from the PDH of *Pandalus borealis* at positions 3, 4, 11, 13, 16, and 17.

INTRODUCTION

Reversible pigment movements within integumental chromatophores and light/dark-adaptive pigment migration in certain ommatidial cells of crustaceans are controlled by neurosecretory hormones (reviews: Kleinholz, 1961; Fingerman, 1970; Rao, 1985). Until recently, knowledge of the primary structure of crustacean pigmentary-effector hormones was restricted to two hormones (Kleinholz, 1985): red pigment concentrating hormone (RPCH), an octapeptide (Fernlund and Josefsson, 1972; Fernlund, 1974a, b), and light-adapting distal retinal pigment hormone (DRPH), an octadecapeptide, isolated from eyestalks of *Pandalus borealis* (Fernlund, 1971, 1976, 1977). The DRPH is one of the several forms of a molecule that acts both as a light-adapting hormone and chromatophorotropic pigment-dispersing hormone, PDH (Kleinholz, 1970, 1975; Riehm and Rao, 1982; Josefsson, 1983).

The structural basis of the heterogeneity of PDH (=DRPH) within any given species remains unknown, but progress is being made in evaluating the structural relationships among the PDHs from different species (Newcomb, 1983; Keller and Kegel, 1984; Quackenbush and Fingerman, 1984; Newcomb *et al.,* 1985). Recently Rao *et al.* (1985) determined the primary structure of an octadecapeptide PDH isolated from eyestalks of the fiddler crab *Uca pugilator.* The sequenced *Uca* PDH shares the amino-terminal and carboxyl-terminal residues, as well as a hexapeptide core sequence, with *Pandalus* PDH (=DRPH: Fernlund, 1976), but shows residue substitutions at six positions. As part of our continuing efforts to characterize the PDHs of representative

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Abbreviations: PDH, pigment-dispersing hormone (also called DRPH, light-adapting distal retinal pigment hormone); RPCH, red pigment concentrating hormone; TDE, thiodiethanol.

species of Crustacea, this report deals with the purification and sequence analysis of the major form of PDH from eyestalks of the crab *Cancer magister.*

Materials and Methods

Eyestalks and animals

Eyestalks, freshly removed from male *Cancer magister* and frozen at −20°C, were supplied by a dealer at whose plant the living crabs were prepared for market. Upon arrival at the laboratory the eyestalks were lyophilized and stored at −20°C in tightly stoppered plastic bottles. Specimens of *Uca pugilator,* utilized in bioassays, were collected at Panacea, Florida, or obtained through a supplier from Panama City, Florida.

Initial extraction and partition

The initial extractions, with all solutions containing 0.1% of thiodiethanol (TDE), were done as described by Fernlund (1971). Powdered lyophilized eyestalks (100 g, about 1250 eyestalks) were heated to near boiling for 5 min with 99% ethanol:0.1 *M* NH_3, 700:300, v/v; the filter cake was washed three times with 200 to 300 ml portions of the same solvent. The combined filtrates, after reduction to about 50 ml in a rotary evaporator, were diluted with water to 100 g and were extracted four times with 100 ml portions of the upper phase of n-butanol-0.1 *M* NH_3, 1:1, v/v. The combined butanol phases, taken to near dryness by rotary evaporation, were dissolved in 0.2 *M* HCl and then partitioned with cyclohexane-butanol, 4:1, v/v (Fernlund, 1971). The HCl phase was dried in a rotary evaporator.

In scaled-up extractions, 250 g of powdered eyestalks were mixed with 900 ml of boiling distilled water and heated for 10 min in a boiling water bath. The mixture was cooled in an ice-water bath and under running tap water to room temperature. Glacial HOAc was added slowly and with stirring to pH 5, and 1 ml of TDE added. The suspension was centrifuged, and the residue extracted six times after stirring for 1 to 3 hours each with 250 ml portions of 0.5 *M* HOAc. The combined supernatants were divided into two portions, each was reduced in volume by rotary evaporation and lyophilized. The lyophilized residues were then subjected to Fernlund's extraction described above, starting with (unheated) 99% ethanol-0.1 *M* NH_3 and proceeding through separation of the HCl phase.

Chromatographic purification

The details of chromatographic conditions are given in the legends to figures and an outline of the protocol is presented here. The dried HCl phase (obtained as above) was extracted with aliquots of 1 *M* HOAc, centrifuged to remove any sediment, and filtered through a column of Sephadex G-25 fine with 1 *M* HOAc containing 0.1% TDE as the eluant. The PDH zones from two preparations filtered on Sephadex G-25 were combined and subjected to cation exchange chromatography on CM Sephadex C-25 utilizing ammonium acetate buffers (containing 0.02% TDE). Accumulated TDE was removed from the combined fractions constituting the major peak of PDH activity by filtering the dissolved residues on columns of Sephadex G-25-superfine, with 0.02 *M* ammonium bicarbonate containing 0.02% TDE as eluant, instead of heating to 50°C under vacuum as described by Fernlund (1971). The PDH zones processed as above from several preparations (from 1.181 kg of eyestalks) were pooled and further purified by partition chromatography on Sephadex G-50-superfine and by reversed-phase high performance liquid chromatography (HPLC) on a Whatman Partisil-10 ODS-2 column as described by Rao *et al.* (1985).

Amino acid composition and sequence analysis

Peptide samples were hydrolyzed *in vacuo* with constant boiling HCl for 22 h at 110°C, and analyzed by Beckman 119CL Analyzer. Sequencing analysis was carried out by stepwise Edman degradation, using a gas-phase automated sequencer (Applied Biosystems, Model 470A), coupled with HPLC identification of phenylthiohydantoin (PTH)-amino acids. Identification of the carboxyl-terminal residue as alanine was followed by a study to determine whether it occurs as Ala-NH_2. For this purpose 1.68 nmol of purified PDH was treated with 0.2% trifluoroacetic acid for 3 h in a boiling water bath (Tarr, 1985), so as to cleave peptide bonds involving Asp (Asp-Ala here). Then the sample was dried, reacted with phenylisothiocyanate, and compared by HPLC with authentic PTC (phenylthiocarbamyl)-Ala and PTC-Ala-NH_2.

Synthetic peptides

Synthetic preparations of *Pandalus* PDH (=DRPH; Riehm and Rao, 1982) and *Uca* PDH (Rao *et al.*, 1985) served as reference compounds for comparison by HPLC or by bioassay.

Bioassay

Solutions of purified and synthetic peptides, as well as aliquots of the fractions from purifications, were assayed for melanophore pigment dispersion in destalked (eyestalkless) fiddler crabs. Samples to be assayed were dissolved in physiological saline (Pantin, 1934). Unless otherwise stated, the injected volume was 50 μl/crab. The stages of melanophore pigment distribution were examined microscopically and scored according to the five-point scale of Hogben and Slome (1931). The observed pigment-dispersing responses were quantified in terms of activity values, as described by Kleinholz and Kimball (1965) and Fingerman *et al.* (1967).

Results

When the HCl fraction of eyestalk extract, resulting from initial extraction and partition, was filtered through a column of Sephadex G-25 fine, the PDH zone emerged at about 0.53 column volume (Fig. 1). Upon CM-Sephadex chromatography several peaks of PDH activity could be resolved, of which the major peak (fractions 26–33) eluted at about one column volume (Fig. 2) and accounted for 66% of the recovered activity. When this material was purified by partition chromatography on a column of Sephadex G-50 superfine, two zones of PDH activity were evident (Fig. 3). The major zone (eluting at 0.74 column volume) was further purified by reversed-phase HPLC (Fig. 4). In this chromatography PDH activity was mainly associated with the UV-peak having a retention time of 14.5 min. Synthetic PDH of *Uca* displayed an identical retention time (14.5 min), whereas *Pandalus* PDH emerged slightly earlier, 13.8 min (Fig. 4).

Quantitative amino acid analysis of a sample of the HPLC-purified PDH indicated that the yield from 1.181 kg of eyestalk powder (about 14,750 eyestalks from *Cancer magister*) was 12.2 nmol (24 μg). This accounted for about 4% of the original activity as assayed by melanophore pigment dispersion in destalked *Uca*.

Amino acid analysis (1.87 nmol sample) showed that the purified PDH of *Cancer* has 18 residues: Asx, 3.61 (4); Ser, 2.03 (2); Glx, 1.31 (1); Pro, 1.36 (1); Gly, 1.44 (1); Ala, 1.32 (1); Val, 1.04 (1); Met, 0.84 (1); Ile, 1.56 (2); Leu, 2.49 (3); Lys, 0.93 (1).

Automated gas phase sequencing (1.68 nmol sample) enabled the assignment of the sequence of residues 1 to 18 (Fig. 5). Since cycle 19 was blank, Ala^{18} can be

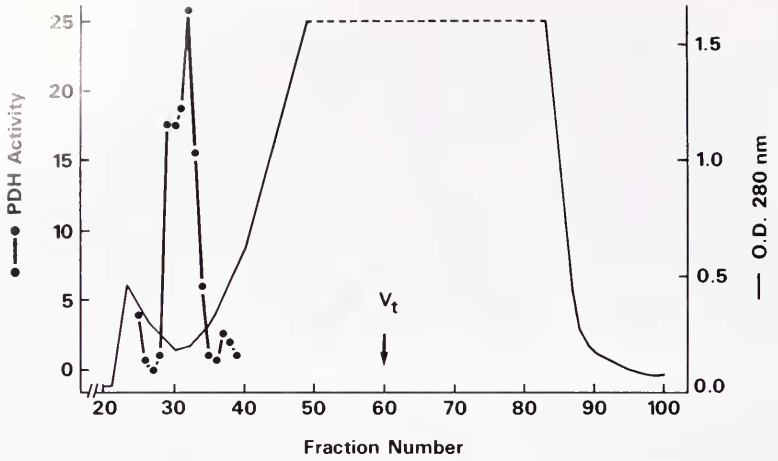

FIGURE 1. Gel filtration of extract of 100 g (1250 eyestalks) of *Cancer magister* on Sephadex G-25 fine. Initial extraction and partition procedures were as described by Fernlund (1971). Column: 2.5 × 92.7 cm; eluant: 1 M HOAc; flow rate: 44.4 ml/h; fraction size: 7.4 ml; V_t: total column volume, 448 ml. Bioassay: 5 μl of a fraction were mixed with 200 μl of Pantin's saline and 25 μl of the mixture was injected into each of 3 destalked *Uca pugilator*. The PDH effect is expressed as activity values (Kleinholz and Kimball, 1965).

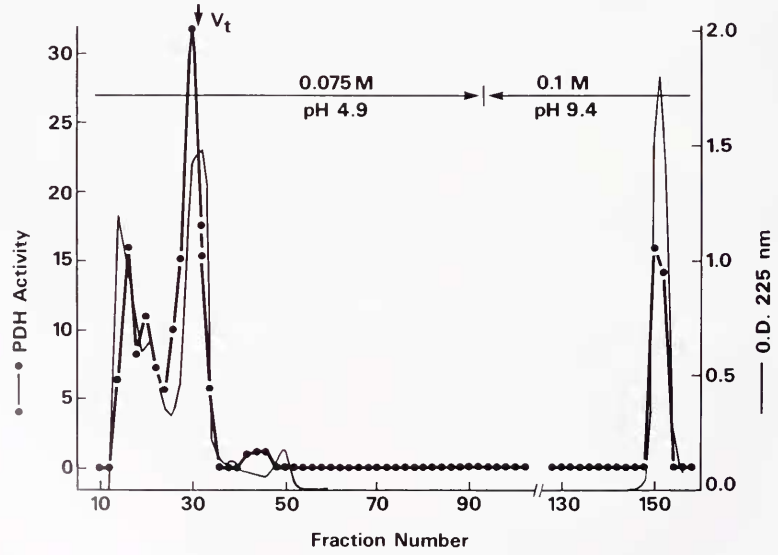

FIGURE 2. Cation-exchange chromatography on CM Sephadex C-25 of the PDH zones (fractions 29–33, Fig. 1) from two preparations filtered on G-25 Sephadex (extract of 223 g of lyophilized eyestalk powder). Column: 0.9 × 106 cm; V_t: total column volume, 67.5 ml; eluant: 0.075 *M*, pH 4.9 ammonium acetate; fractions of 2.8 ml were collected every 12.7 min to fraction 92; the head of buffer above the gel was then replaced with 0.1 *M*, pH 9.4 ammonium acetate and step-wise elution with this buffer continued, fractions of 2.8 ml being taken every 12.7 min. Bioassay: aliquots of 20 μl from alternate fractions from the column were lyophilized, the residue dissolved in 350 μl Pantin's saline, and injected into 4 destalked *Uca* (50 μl/crab). The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967).

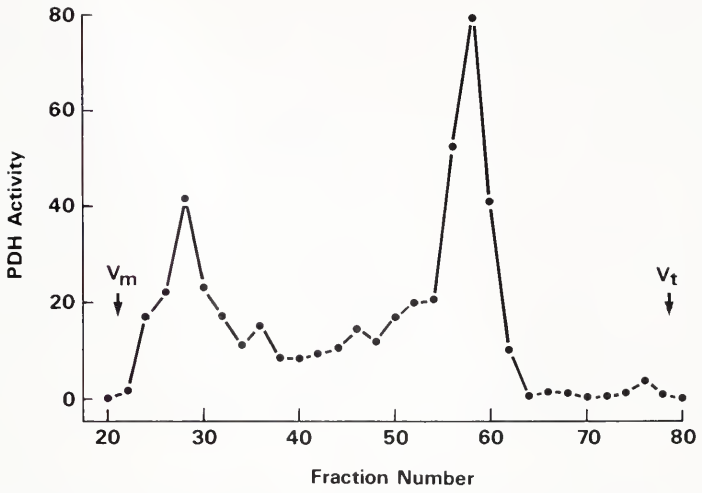

FIGURE 3. Partition chromatography on Sephadex G-50 superfine of the combined major PDH zones obtained by cation-exchange chromatography (fractions 26–33 in Fig. 2) from 1.181 kg of eyestalk powder. Column: 1.5 × 88.5 cm; eluant (without TDE): organic phase of n-butanol-HOAc-H_2O, 4:1:5, v/v; flow rate of 3.9 ml/h, fraction size 2.0 ml; V_m, volume of mobile phase, 44.5 ml; V_t, total volume of column, 156 ml. Bioassay: 5 μl aliquots from alternate fractions were lyophilized, the residues dissolved in 0.35 ml saline, and 50 μl injected into each of 3 test *Uca*. The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967).

regarded as the carboxyl-terminal residue. Further characterization of this residue, by HPLC analysis of the products of dilute acid cleavage, showed that the carboxyl-terminal Ala occurs in an amidated form. Thus the primary structure of the purified PDH from *Cancer magister* is: Asn-Ser-Glu-Leu-Ile-Asn-Ser-Ile-Leu-Gly-Leu-Pro-Lys-Val-Met-Asn-Asp-Ala-NH_2. A synthetic preparation of this octadecapeptide (Rao *et al.*, 1985) and the purified native PDH from *Cancer magister* were equipotent in assays for melanophore pigment dispersion in destalked *Uca pugilator* (Fig. 6).

DISCUSSION

The present study shows that the PDH purified from eyestalks of *Cancer magister* is structurally identical to the major form of PDH in eyestalks of *Uca pugilator* (Rao *et al.*, 1985). For convenience in discussion this octadecapeptide will be hereafter called β-PDH, whereas the first characterized PDH (=DRPH) from *Pandalus borealis* (Fernlund, 1976) will be designated as α-PDH. The amino acid sequences of the two PDHs show 66.7% identity, the differences being found at positions 3, 4, 11, 13, 16, and 17, as shown below:

α-PDH: Asn-Ser-^{3}Gly-Met-^{5}Ile-Asn-^{7}Ser-Ile-^{9}Leu-Gly-^{11}Ile-Pro-^{13}Arg-Val-^{15}Met-Thr-^{17}Glu-Ala-NH_2

β-PDH: Asn-Ser-*Glu*-*Leu*-Ile-Asn-Ser-Ile-Leu-Gly-*Leu*-Pro-*Lys*-Val-Met-*Asn*-*Asp*-Ala-NH_2

Besides sharing the amino and carboxyl-terminal residues, the two PDHs have a common hexapeptide core sequence (residues 5–10: Ile-Asn-Ser-Ile-Leu-Gly). Whether these residues comprise the "message sequence" of the PDH remains unknown, although studies with synthetic truncated analogs point to the importance of residues 6–9 for PDH activity (Riehm and Rao, 1982; Riehm *et al.*, 1985).

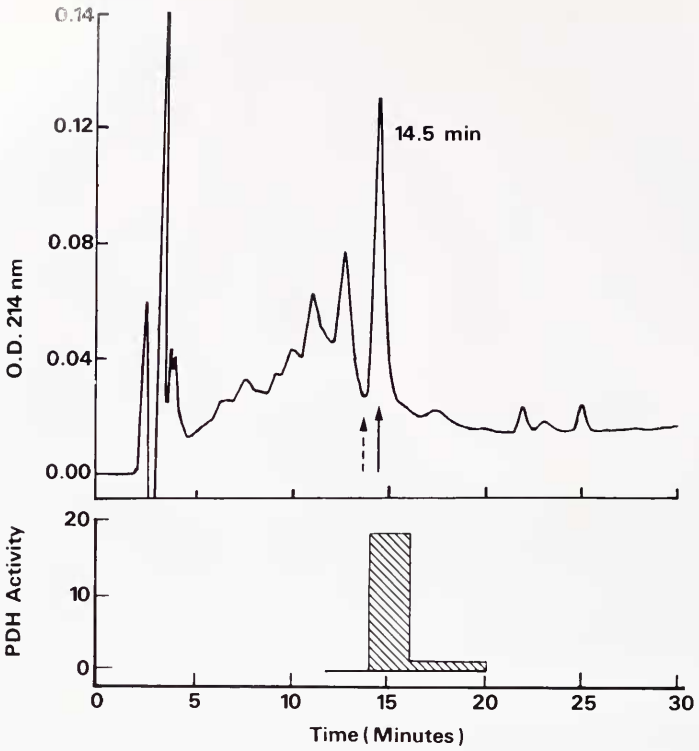

FIGURE 4. The major active zone from partition chromatography (fractions 55–60, Fig. 3) was subjected to reversed-phase HPLC on a column of Partisil-10 ODS-2 (4.6 mm × 25 cm, Whatman) utilizing Waters Liquid Chromatography System (Data Module 730; Programmable Controller 721; Pumps 510; Absorbance Detector 441). Solvent system: linear gradient elution for 30 min from 30% CH_3CN to 45% CH_3CN, both with 0.1% trifluoroacetic acid; flow rate, 1 ml/min. Bioassays were done with 0.05% of each fraction; each aliquot was lyophilized and dissolved in 0.5 ml saline; injected 50 μl/crab into 5 test *Uca*. The reference compounds utilized were: synthetic PDH of *Pandalus borealis* (Riehm and Rao, 1982), retention time 13.8 min (broken arrow); and synthetic PDH of *Uca* (Rao *et al.*, 1985), retention time 14.5 min (solid arrow). The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967).

Studies involving paper electrophoresis (Stephens *et al.*, 1956; Fingerman, 1966), disc electrophoresis (Rao *et al.*, 1970; Keller, 1977), ion-exchange chromatography (Rao *et al.*, 1970; Fernlund, 1971; Kleinholz, 1970, 1972; Dores and Herman, 1980), and reversed-phase HPLC (Keller and Kegel, 1984) showed that multiple forms of PDH are present in extracts of eyestalks or sinus glands of diverse decapod Crustacea. Initial resolution of the multiple forms of PDH can be best achieved, however, by ion-exchange chromatography (Kleinholz, 1970; Rao *et al.*, 1985). Since the PDHs separable by ion-exchange chromatography display identical elution profiles in gel filtration, the multiple forms of PDH are thought to differ in net charge but not in molecular size (Kleinholz, 1972). This view is supported by the finding that the two sequenced PDHs are both octadecapeptides that differ in net charge. The possibility that some of the variants are products of amino-terminal truncation, resulting from enzymatic breakdown, merits exploration.

The presence of Glu^3 (rather than Gly^3) renders β-PDH more acidic than α-PDH (Rao *et al.*, 1985). Consequently, during cation-exchange chromatography in low ionic strength buffers at pH 4.8–4.9, β-PDH elutes relatively fast, at about one column volume (Dores and Herman, 1980; Rao *et al.*, 1985), whereas α-PDH emerges later,

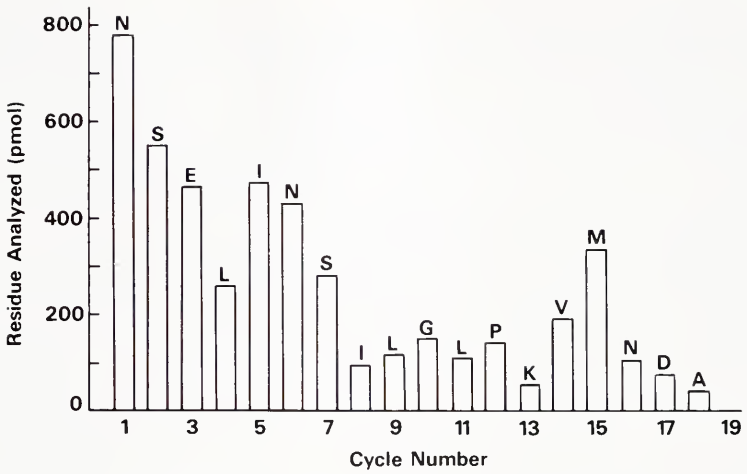

FIGURE 5. Yields of amino acid residues during the sequence analysis of *Cancer* PDH (1.68 nmol load) by stepwise Edman degradation, utilizing a gas-phase automated sequencer (Model 470 A, Applied Biosystems), coupled with HPLC identification of the resulting PTH-amino acids. Nomenclature of amino acids by the one-letter code: A (Ala), D (Asp), E (Glu), G (Gly), I (Ile), K (Lys), L (Leu), M (Met), N (Asn), P (Pro), S (Ser), V (Val).

at about three column volumes (Fernlund, 1971). In extracts of eyestalks from *Uca pugilator*, β-PDH is the major form of dispersing hormone (Rao *et al.*, 1985). A peak corresponding to α-PDH is either not evident (Rao *et al.*, 1985) or may account for

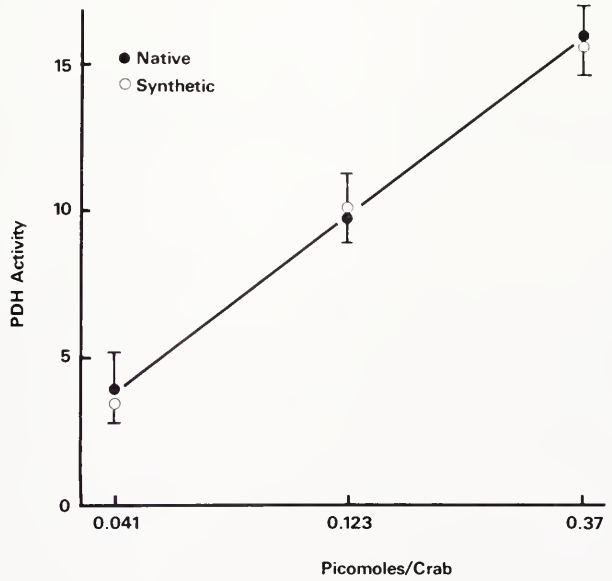

FIGURE 6. Dose-related melanophore pigment-dispersing activity elicited by purified native PDH of *Cancer magister* and a synthetic preparation of this peptide (first called *Uca* PDH; Rao *et al.*, 1985). Each data point (mean, standard deviation) was derived from 3 assays (5 destalked *Uca* per assay). The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967). Pantin's physiological saline (control) did not elicit any dispersion in the melanophores.

<2% of the recovered *Uca* PDH activity in cation-exchange chromatography (Dores and Herman, 1980). β-PDH is the predominant form in *Cancer magister* as well. In eyestalks of *Pandalus borealis* (Fernlund, 1971), however, α-PDH was reported to be the major form. Although two smaller peaks of activity eluting faster than α-PDH in cation-exchange chromatography were found in this species, it is not known whether either of these is identical to β-PDH. Clearly, much remains to be revealed about the structural basis of PDH heterogeneity within a given species and about the molecular interrelationships of PDHs among diverse species.

Although the two sequenced PDHs are both octadecapeptides with 67% identity in primary structure, the occurrence of markedly different PDHs has been reported. For example, Keller and Kegel (1984) found 28–29 amino acid residues in two PDHs purified by HPLC from extracts of sinus glands of the crab, *Eriocheir sinensis*. Quackenbush and Fingerman (1984) isolated a smaller PDH (1386 daltons) with arginine as the amino-terminal residue from eyestalks of *Uca pugilator*. Studies with PDH from sinus glands of *Cardisoma carnifex* have been inconclusive (Newcomb, 1983; Newcomb *et al.,* 1985), but they note that the peptide may have a blocked amino-terminus (unpublished data cited by Newcomb *et al.,* 1985). Since none of these seemingly distinct PDHs has been sequenced, their relationship to α-PDH and β-PDH remains unclear. It is of comparative interest that variation in molecular size as well as differences in net charge are evident among the melanophore-stimulating hormones (MSHs) of vertebrates (Li, 1978; Baker, 1979; Hruby *et al.,* 1984).

Both variation in chain length and residue substitutions are evident among arthropod peptides related to crustacean RPCH (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2; Fernlund and Josefsson, 1972). Thus, the related AKH (adipokinetic hormone) from locust is a decapeptide (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2; Stone *et al.,* 1976), whereas the related myotropic/cardioacceleratory hormones from cockroach are octapeptides: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH_2 and pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2 (Scarborough *et al.,* 1984; Witten *et al.,* 1984).

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